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# The proprotein convertase *amontillado* (*amon*) is required during *Drosophila* pupal development

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ABSTRACT

Peptide hormones governing many developmental processes are generated *via* endoproteolysis of inactive precursor molecules by a family of subtilisin-like proprotein convertases (SPCs). We previously identified mutations in the *Drosophila amontillado* (*amon*) gene, a homolog of the vertebrate neuroendocrine-specific Prohormone Convertase 2 (PC2) gene, and showed that *amon* is required during embryogenesis, early larval development, and larval molting. Here, we define *amon* requirements during later developmental stages using a conditional rescue system and find that *amon* is required during pupal development for head eversion, leg and wing disc extension, and abdominal differentiation. Immuno-localization experiments show that *amon* protein is expressed in a subset of central nervous system cells but does not co-localize with peptide hormones known to elicit molting behavior, suggesting the involvement of novel regulatory peptides in this process. The *amon* protein is expressed in neuronal cells that innervate the corpus allatum and corpora cardiaca of the ring gland, an endocrine organ which is the release site for many key hormonal signals. Expression of *amon* in a subset of these cell types using the GAL4/UAS system in an *amon* mutant background partially rescues larval molting and growth. Our results show that *amon* is required for pupal development and identify a subset of neuronal cell types in which *amon* function is sufficient to rescue developmental progression and growth defects shown by *amon* mutants. The results are consistent with a model that the *amon* protein acts to proteolytically process a diverse suite of peptide hormones that coordinate larval and pupal growth and development.



## Introduction

Many peptide hormones and neuropeptides are produced by limited proteolysis of inactive precursor peptides. The release of an active peptide hormone from its precursor molecule typically occurs by proteolytic cleavage after paired basic amino acids and is mediated by a family of proteases known as the subtilisin-like proprotein convertases (SPCs) (Bergeron et al., 2000). Following convertase processing, most peptides undergo removal of the C-terminal basic residues *via* a carboxypeptidase (Fricker and Leiter, 1999) and subsequent α-amidation of the C-terminal glycine residue (Kulathila et al., 1999). Processing of precursor proteins may liberate a single or multiple bioactive products (Sossin et al., 1989; Zhou et al., 1999), and a given precursor may be differentially processed in a cell-specific fashion depending on the SPC processing enzymes expressed (Furuta et al., 1997; Rouille et al., 1995). Thus processing of peptide hormone precursors may serve as an important regulatory step to modulate peptide and neuropeptide signaling.

Prohormone Convertase 2 (PC2) is one of seven vertebrate SPCs identified to date and displays a neuroendocrine expression pattern that implicates the enzyme in the activation of peptide hormones and neuropeptides (Muller and Lindberg, 2000). The *Drosophila* PC2 homolog, *amontillado* (*amon*), was identified based on sequence similarity to conserved regions between yeast Kex2, human furin, and human PC2 (Siekhaus and Fuller, 1999). *amon* is expressed throughout the life cycle of the fly, and *amon* transcripts localize to the larval central nervous system and regions of the gut (Siekhaus, 1997; Siekhaus and Fuller, 1999), suggesting that *amon* acts in neuroendocrine tissues. The *amon* protein has been shown to be an active protease on a KR containing synthetic peptide when expressed in *Drosophila* S2 cells with the *Drosophila* 7B2 protein, a homolog of the 7B2 protein that functions in maturation of vertebrate PC2 (Hwang et al., 2000). Isolation and analysis of a series of EMS induced *amon* mutants showed that *amon* is required during embryogenesis and early larval development and suggests that the *amon* protein may act to process peptide hormones that control hatching, larval growth, and larval molting (Rayburn et al., 2003).

The regulation of molting and metamorphosis in insects has its roots in an endocrine axis and relies on a delicate interplay between steroid and peptide hormones (Ewer, 2005; Mesce and Fahrbach, 2002; Nijhout, 1994; Riddiford, 1993; Truman, 1992; Zitnan et al., 2007). This axis includes the brain, the corpus allatum (CA), and the prothoracic gland which act as sources of peptide and steroid

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hormones, the effectors of molting and metamorphosis. Pulses of the steroid hormone 20-hydroxyecdysone, hereafter referred to as ecdysone, act to initiate larval molting (Riddiford, 1993). One manner in which ecdysone may exert its regulatory effect on molting and metamorphosis is by affecting the expression of genes whose products are part of the ecdysis cascade. For example, in both *Manduca* and *Drosophila* the peptide hormone gene *ecdysis triggering hormone* (*eth*) contains a putative ecdysone response element upstream of the transcription start site (Park et al., 1999; Zitnan et al., 1999). Studies in *Manduca* show that rising ecdysteroid levels prior to ecdysis induce *eth* gene expression in the Inka cells and that a drop in the ecdysteroid titer is required for ETH release (Zitnan et al., 1999).

Two additional peptide hormones, eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) are known to interact with ETH in an ecdysis cascade to elicit the behavioral outputs that characterize molting and metamorphosis in insects. Low levels of circulating ETH trigger the release of EH from the brain. This initial EH release induces a subsequent and exhaustive release of ETH from the Inka cells which in turn cues exhaustive release of EH (McNabb et al., 1997). In *Manduca*, EH acts through a second messenger system to cause elevated levels of cGMP in cells that express CCAP (Ewer et al., 1997). CCAP release elicits ecdysis motor burst while suppressing the pre-ecdysis behaviors initiated by EH (Gammie and Truman, 1997). Notably, the ETH and CCAP pro-peptides of *Drosophila* contain putative dibasic processing sites (Park et al., 1999, 2003) suggesting they require endoproteolytic activation. The *Drosophila* EH pro-peptide also contains a possible dibasic processing site (Horodyski et al., 1993), although processing at this site may not be necessary for production of the bioactive EH peptide.

Here we have used a conditional rescue system to ask whether *amon* is required during postembryonic developmental transitions in *Drosophila*. *amon* mutants rescued past earlier embryonic and larval requirements by heat-shock induced expression of an *amon* cDNA and then removed from the rescue regime at the late third instar stage display defects in head eversion, leg and wing extension, and abdominal differentiation, indicating that *amon* activity is required for these aspects of metamorphosis. Although *amon* mutants show similar phenotypes to *eth* mutants and to pupae resulting from CCAP-cell ablation, the *amon* protein does not co-localize with ETH, CCAP, or EH, suggesting the existence of novel peptide hormones that regulate molting and metamorphosis. Interestingly, the *amon* protein localizes to cells that innervate the CA and corpora cardiaca (CC) sections of the ring gland, suggesting that *amon* protein may regulate the endocrine activity of this gland. Finally, expression of *amon* in a subset of neuronal cell types in an *amon* mutant background is sufficient to partially rescue developmental progression and growth.

## Materials and methods

### *Conditional rescue of amon mutants*

One hundred fifty *yw; hs-amon/hs-amon; Df(3R)Tl-X e/TM3 Sb Ser* $y^+$ *e* virgin females were crossed to 150 *yw;* $amon^{C241Y}$ or $amon^{Q178st}$ *e/TM3 Sb Ser* $y^+$ *e* males in an egg collection chamber and allowed to lay eggs on 100 mm grape juice agar plates spotted with yeast paste for 3 days at 25 °C. On the third day, a 4 or 8 h egg collection was taken and *yellow* larvae (*yw; hs-amon/+;* $amon^{C241Y}$ or $amon^{Q178st}$ *e/Df(3R)Tl-X e*) were collected at 36 h after egg laying (ael) and placed on a fresh plate. Animals were heat shocked for 30 min at 37 °C every 24 h beginning at 36 h ael and subsequently scored for survival until all the animals on the positive control plate eclosed. Experimental animals were heat shocked as above until 108 h ael. After each scoring, larvae were transferred to a fresh plate unless the animal had pupariated. Dead larvae were mounted in polyvinyl lactophenol for examination. Pupae from the experimental plate were removed from the pupal case in water under a Leitz dissecting scope once a majority of the control animals had successfully eclosed. Once out of the pupal case, animals were photographed using a digital camera (Hamamatsu 3CCD) mounted to a Leitz dissecting scope. Control animals failing to eclose were also removed from the pupal case and photographed at later time points. Percent pupariated and eclosed values were calculated by dividing the number of animals that had pupariated or eclosed by the number of animals collected at 36 h ael.

### *Antibody development*

A peptide corresponding to the final 27 amino acids of the *amon* protein with three additional amino terminal residues (CKC) was synthesized by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. The peptide was coupled *via* M-Maleimidobenzoyl N-Hydroxysuccinimide Ester (MBS, Pierce) to bovine serum albumin as described below and then injected into five mice at the Monoclonal Antibody Facility at the University of Georgia. Following an initial subcutaneous injection with Freund's complete adjuvant (Sigma Chemical), mice were boosted intraperitoneally using Freund's incomplete adjuvant every 21 days and bleeds were taken approximately 7 days after each boost. Beginning with the third bleed, serum was tested *via* Western analysis for its ability to recognize the *amon* protein. Following the sixth boost one mouse produced serum specific for *amon* protein and was subsequently boosted a total of 15 times over the course of about 1 year. Bleeds taken after the seventh boost were used in the immunocytochemistry and Western analyses as described below.

### *Antigen generation*

Five milligrams of carrier protein (Bovine Serum Albumin, Sigma) was dissolved in 1 mL of phosphate buffered saline (PBS). 50 μL of MBS in N, N dimethylformamide (DMF) was added drop by drop to 500 μL of the carrier protein solution and then stirred at room temperature for 30 min. Excess MBS was removed using Micro Bio-Spin P-30 Tris Chromatography Columns (Bio-Rad) equilibrated with PBS. A 20-fold excess of synthetic peptide was added to the carrier protein–MBS solution and stirred at room temperature for 3 h. To make sure that peptide was coupled to the carrier protein, the solution was run on a 10% polyacrylamide gel next to a control solution (carrier protein–MBS solution alone) and stained with Coomassie.

### *Immunocytochemistry*

Wandering third instar larvae (*Canton S*) were dissected, fixed (2 h in PBS and 175 mM NaCl, pH 7.4, with 4% paraformaldehyde), dehydrated, and rehydrated according to the protocol of Cao and Brown (2001). Tissues were washed in TBS (25 mM Tris, 137 mM NaCl, 0.27 mM KCl) containing 0.5% Triton X-100 (TBST) two times for 5 min each and then blocked in TBST+ 5% goat serum overnight at 4 °C. All subsequent steps were performed at 4 °C. Primary antiserum was added in TBST+ 2% BSA at a 1/200 dilution and left overnight. Following a wash of at least 1 h in TBST+ 2% BSA, the secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488, 568 or 594, Molecular Probes, Inc.) was added at a dilution of 1:500–1:2000 in TBST+ 2% BSA and tissues were incubated overnight in the dark. Tissues underwent a final wash in TBST and were then mounted on slides in a 1:1 TBST/glycerol solution. Images were captured on an Olympus BX60 microscope using a JVC digital camera (model KYF70BM) and the AutoMontage (Syncroscopy) software. To obtain *amon* mutant third instar CNS tissues for use as a control, *amon* mutant larvae (*yw; hs-amon/+;* $amon^{Q178st}$ *e /Df(3R)Tl-X e*) were rescued past early developmental requirements for *amon* function by periodic expression of a *hs-amon* transgene for 45 min at 37 °C every 24 h, beginning at 36 h AEL until 84 h AEL. The CNS was dissected at

108 h AEL (24 h after the final 84 h AEL heat-shock) from both *amon* mutants and control siblings (*yw; hs-amon/+; Df(3R)Tl-X e* or *amon$^{Q178st}$ e/TM3 Sb Ser y+ e*).

For co-localization of *amon* and ETH, the same protocol was followed except that after blocking, tissues were incubated with the mouse antiserum directed against the *amon* peptide for 3 h, washed for 1 to 2 h, incubated in the anti-mouse secondary antibody for 3 h, and washed overnight. The next day, the same procedure was followed for a rabbit antiserum directed against ETH (Park et al., 2002) used at a 1:500 dilution and a secondary antibody (anti-rabbit IgG conjugated to Alexa Fluor 568, Molecular Probes, Inc.) used at a 1:500 dilution. Alternatively, tissues were incubated in the mouse anti-amon antiserum for 3 h, washed for 1 to 2 h, incubated in the rabbit anti-ETH antiserum for 3 h, washed for 1 to 2 h, and then incubated in both mouse and rabbit secondary antibodies overnight. The following day, tissues were washed for 3 h in TBST, mounted on slides, and visualized.

For co-localization with tissues expressing GFP, the UAS-mCD8-GFP line (Lee and Luo, 1999) was crossed to EH-GAL4 (McNabb et al., 1997), Kurs-21, (Siegmund and Korge, 2001), and CCAP-GAL4 (Park et al., 2003) to obtain progeny with GFP in the EH neurons, the CA-innervating neurons, and the CCAP neurons respectively. Wandering third instar larvae were dissected and then processed as described above for *amon* protein detection.

### *Western analysis*

Animals were homogenized in cracking buffer (0.125 M Tris–HCl, pH 6.7, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 4 M urea) (10 μL per adult fly, 5 μL per third instar larvae) containing a Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche) using motorized hand held pellet pestles (Konte) for 1.5 mL eppendorf tubes. Protein homogenates were boiled for 5 min and centrifuged for 10 min at 14,000 ×*g* using a benchtop microfuge. The supernatant was boiled for 5 min in an equal volume of sample buffer (0.125 M Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate, 20% glycerol, and 0.02% bromophenol blue) and then loaded onto a 10% polyacrylamide gel.

Proteins were transferred to a nitrocellulose membrane (Schliecher and Schuell BioScience, Inc) overnight in a Bio-Rad Transfer Cell and the membrane was blocked in blocking solution (10% non-fat dry milk, 30% goat serum) in TBS/Tween (20 mM Tris–HC1, pH 7.5, 500 mM NaCl, 0.05% Tween20) overnight at −20 °C. Primary antiserum was added at a 1:2000 dilution in blocking solution and incubated at room temperature for 2 to 3 h. After three washes of at least 5 min each, a horseradish peroxidase conjugated secondary antibody (anti-mouse IgG-HRP, Promega) was added at a 1:1500 dilution and incubated for 30 min at room temperature. Following three more washes of at least 5 min each, peroxidase activity was detected using Lumi-Light Western Blotting Substrate (Roche). Excess substrate solution was blotted off the membrane and then images were captured using Kodak BioMax Light film.

For the developmental Western analysis, a 4 h *Canton-S* egg collection was taken at 25 °C and animals were aged to the following time points after the end of egg laying (ael): 16 h, 20 h, 40 h, 64 h, 84 h, and 96 h. In our hands these time points correspond to embryogenesis (16 and 20 h ael), first instar, second instar, early third instar, and mid-third (non-wandering) instar, respectively. For the wandering third instar and white pre-pupal time points, animals were collected at the appropriate developmental stage. Animals were homogenized as described above in the following amounts of cracking buffer: 0.25 μL per first or second instar animal, 3 μL per early third instar animal, 5 μL per mid and wandering third instar animal, and 10 μL per white pre-pupa.

For antibody blocking experiments, approximately 100 μg of the C-terminal *amon* peptide used for injection into mice, or one of two control peptides corresponding to EcR-B1 or EH, was added to the antiserum in 1 mL of blocking solution and then allowed to incubate for 30 min at room temperature. The consequences of pre-absorption with *amon* or control peptides were then tested using a Western blot as an assay.

### *Rescue of amon mutant defects*

To determine if *amon* function in specific neuronal cell types is sufficient to rescue *amon* mutant defects, approximately 150 *yw; uas-amon$^{40L}$; Tl-X e/TM3 Sb Ser y+ e* virgin females and 150 *yw; +; Kurs21-gal4, amon$^{C241Y}$ e/TM3 Sb Ser y+ e* males were mated in an egg collection chamber containing a grape juice agar plate with fresh yeast paste at 25 °C. As a control cross, 150 *yw; uas-amon$^{40L}$; Tl-X e/TM3 Sb Ser y+ e* virgin females and 150 *yw; +; amon$^{C241Y}$/TM3 Sb Ser y+ e* males were mated in the same way. A 4 h egg collection on grape juice agar plates with yeast paste was taken 24 h later. At 36 h ael *amon* mutant larvae, recognizable by the *yellow* marker, (*yw; uas-amon$^{40L}$/+; Tl-X e/Kurs21-gal4, amon$^{C241Y}$ e*) and wild-type control larvae (*yw; uas-amon$^{40L}$/+; Tl-X e* or *amon$^{C241Y}$ e/TM3 Sb Ser y+ e*) were selected and moved to fresh grape juice agar plates with yeast paste. Larvae were scored every 24 h based on spiracle morphology to determine whether they were first, second, or third instar larvae.

## Results

### *amon is required during pupal development*

*amon* mutants arrest during embryogenesis and early larval development (Rayburn et al., 2003). To determine the requirements for *amon* during later development, we have rescued *amon* mutants by periodic heat induced expression of an *amon* cDNA. In Table 1, we show that continuous daily expression of *amon* (see Materials and methods) starting at 36 h after egg laying (ael) rescues two *amon* mutant hemizygotes, *amon$^{C241Y}$* and *amon$^{Q178st}$*, to adulthood. In contrast, removal of heat-shock treatment at the late third instar stage (108 h ael) results in a failure to eclose.

As shown in Figs. 1C–F, the predominant phenotype of the arrested *amon$^{C241Y}$* animals was a failure to evert the head coupled with a failure to extend the legs and wings (91%, Table 2). This phenotype also predominated in arrested *amon$^{Q178st}$* animals (33%, Table 2). Interestingly, the failures in head eversion and leg and wing extension did not preclude further development of these structures as evidenced by tanning and bristle formation (Figs. 1C,D), nor did they prevent the differentiation and pigmentation of the eye (data not shown). Because events such as bristle formation on the legs and eye pigmentation typically occur after a successful head eversion (Bainbridge and Bownes, 1981), the phenotypes of the *amon* mutant pupae indicate an uncoupling of the early event of head eversion and the events characteristic of late stages of pupal development.

A smaller proportion of *amon* mutants displayed failures in abdominal differentiation (Figs. 1C,D, Table 2). For example, 58% of the *amon$^{C241Y}$* animals and 27% of the *amon$^{Q178st}$* did not complete abdominal differentiation. The undifferentiated abdomens resembled

**Table 1**
*amon* is required for eclosion.

| Genotype | Treatment | % Eclosed |
|---|---|---|
| *yw; hs-amon/+;amon$^{C241Y}$/Tl-X* | Control | 42 ($n=50$) |
| *yw; hs-amon/+;amon$^{C241Y}$/Tl-X* | Experimental | 1 ($n=71$) |
| *yw; hs-amon/+;amon$^{Q178st}$/Tl-X* | Control | 60 ($n=15$) |
| *yw; hs-amon/+;amon$^{Q178st}$/Tl-X* | Experimental | 0 ($n=22$) |

*amon* mutant hemizygotes, recognizable by the *yellow* marker, were collected at 36 h ael and were heat shocked every 24 h until all animals eclosed (control) or until the late third instar stage (experimental). *n* equals the number of mutant hemizygotes collected at 36 h ael and % eclosed equals the number of animals eclosed/*n*. *Tl-X, Df(3R)Tl-X*, is a deficiency that removes *amon* (Rayburn et al., 2003).

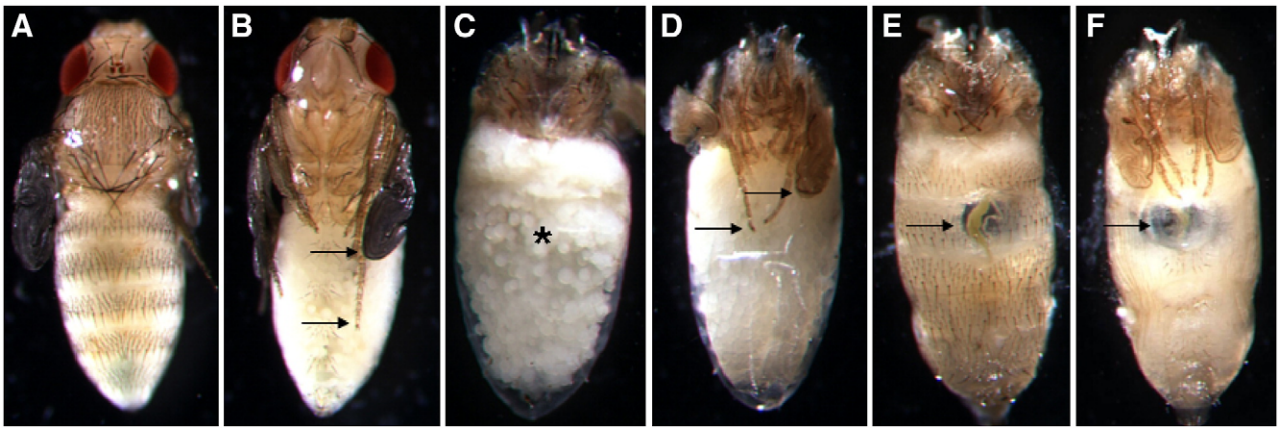


**Fig. 1.** *amon* is required during pupal development. Dorsal (A) and ventral (B) views of a wild-type (*Canton S*) stage P13 pupa showing completion of head eversion, leg and wing extension (arrows), and abdominal differentiation. Dorsal (C) and ventral (D) views of an *amon* mutant (*yw; hs-amon/+; amon$^{C241Y}$/Df(3R)Tl-X*) rescued by *amon* expression during larval development. In this animal, head eversion is blocked and leg and wing extension is defective (arrows). In addition, abdominal differentiation has failed to take place (asterisk). Dorsal (E) and ventral (F) views of a second *amon* mutant (*yw; hs-amon/+; amon$^{C241Y}$/Df(3R)Tl-X*) rescued by *amon* expression during larval development. Arrows indicate the gas bubble that has failed to translocate to the posterior end of the puparium during metamorphosis.

those of stage P5 (Bainbridge and Bownes, 1981) which are transparent and lack segmentation and bristles. The failure in abdominal differentiation did not, however, prevent the thoracic segment from acquiring pigment and bristles, indicating that these two body segments are governed by distinct developmental controls. An additional phenotype displayed by 15% of the *amon$^{C241Y}$* mutants was a failure to displace the pre-pupal gas bubble, an event that typically marks the transition from the P2 to the P4 stage of development (Bainbridge and Bownes, 1981) (Figs. 1E,F, Table 2). Interestingly, all of the pupae failing to displace the mid-body gas bubble developed bristled abdomens, a characteristic of a much later developmental stage (P12i) (Bainbridge and Bownes, 1981). Collectively, our results show that *amon* is required for successful coordination and completion of pre-pupal and pupal development.

### *The amon protein is expressed during pupal stages*

To determine the localization pattern of the *amon* protein, we developed a mouse polyclonal antibody against the *amon* protein and used it in Western and immunocytochemistry experiments to evaluate the presence and localization of the protein. On Western blots, the polyclonal antiserum recognizes a 97 kDa band that is present in all developmental stages examined (Fig. 2). The levels of the 97 kDa protein increase in abundance over developmental time in accordance with RNA expression data reported earlier for *amon* (Siekhaus and Fuller, 1999). This pattern of expression is consistent with the requirements we have uncovered for *amon* during larval and pupal life (Rayburn et al., 2003 and this paper). Recognition of the 97 kDa band on Western blots is reproducibly abolished in blocking experiments (see Materials and methods) in which the *amon* polyclonal antiserum is pre-incubated with the *amon* peptide antigen but not abolished when control peptides corresponding to EcR-B1 or EH are used (data not shown). Recognition of a second 66 kDa band seen at some developmental stages (Fig. 2) is not blocked by pre-incubation with the *amon* peptide. The 66 kDa protein thus appears to be antigenically unrelated to *amon*.

In tissue stains (Figs. 3A,B), we find that the *amon* antiserum recognizes cells within the central nervous system (CNS) of wild-type third instar larvae but that no signal is seen in this tissue in *amon* mutant third instar larvae (see Materials and methods). The localization pattern in the larval CNS consistently includes paired ventral nerve cord cells as well as a pair of brain cells known as the medial neurosecretory cells (MNCs) (Cao and Brown, 2001; Rulifson et al., 2002) or the insulin producing cells (IPCs) (Rulifson et al., 2002). We report elsewhere that the *amon* protein co-localizes with DILP2 in these cells (Rhea, Rayburn and Bender, in preparation). The *amon* antiserum also recognizes cells within the midgut of wild-type third instar larvae. These cells include endocrine cells within thicker portions of the gut, a pattern consistent with *in situ* hybridization experiments using *amon* nucleic acid probes (Siekhaus, 1997). Midgut endocrine cells have been identified in a variety of insect species and are hypothesized to act as monitors of the presence or absence of food in the gut, releasing their peptide hormone contents to direct food movement and digestion (Brown, 2003; Brown and Lea, 1989).

### *Candidate targets of the amon protein*

The homology of *amon* to proprotein convertase genes and the phenotypes of *amon* mutant larvae and pupae suggest that peptide hormones governing larval molting, larval growth, head eversion, leg and wing extension and abdominal differentiation are potential

**Table 2**
Phenotypes of *amon* mutant pupae.

| Phenotype | *amon$^{C241Y}$* ($n=33$) | *amon$^{Q178st}$* ($n=22$) |
|---|---|---|
| Failure to evert head | 91 | 33 |
| Failure to extend legs and wings | 91 | 33 |
| Undifferentiated abdominal cuticle | 58 | 27 |
| Gas bubble not displaced | 15 | 0 |

Failure to evert the head and to extend the thoracic appendages is the predominant phenotype observed in *amon* mutant hemizygotes (*amon/Df(3R)Tl-X*). Additional phenotypes observed include failures in abdominal differentiation and mid-body gas bubble displacement.

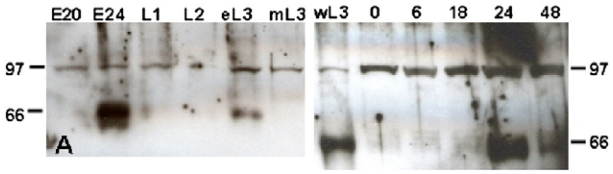


**Fig. 2.** Developmental expression of the *amon* protein. The anti-AMON antiserum detects a 97 kDa band that is present in wild-type (*Canton S*) animals during all stages of embryogenesis, larval development, and pupal development tested. Approximately equivalent amounts of protein were loaded in each lane (see Materials and methods) from the following stages of development: 16 to 20 (E20) and 20 to 24 (E24) hour old embryos, the first (L1) and second (L2) instar larval stages, early (eL3), mid (mL3), and wandering (wL3) third instar stages and pupal stages beginning at pupariation (time 0) and continuing through pupal development 48 h after pupariation (48). The 66 kDa band indicated here appears to be antigenically unrelated to the *amon* protein (see the Results and Materials and methods sections).

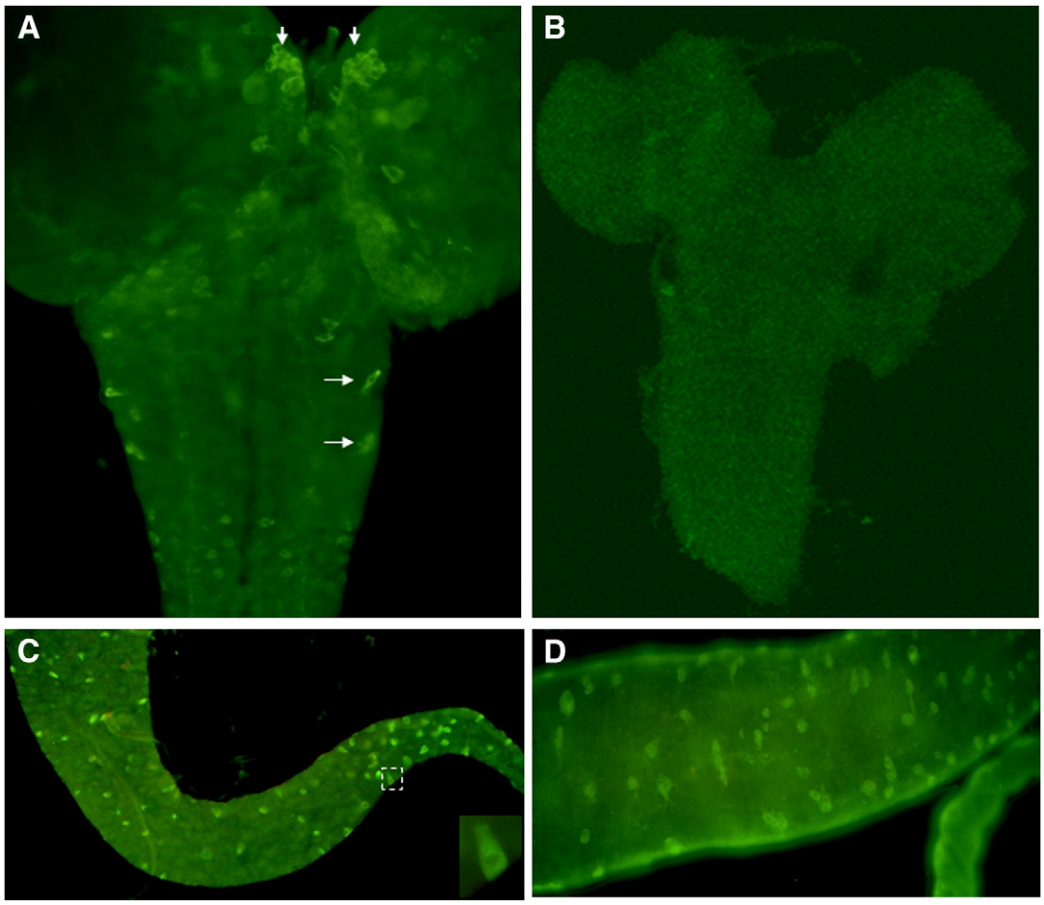


**Fig. 3.** The *amon* protein localizes to the central nervous system and larval midgut endocrine cells. (A) Third instar larval CNS labeled for *amon* protein (20× magnification). The large arrowheads indicate *amon* protein localization to the MNCs. Small arrows indicate prominent ventral nerve cord cells. (B) *amon* mutant third instar larval CNS shows a lack of staining using the *amon* antiserum (20×). (C) Third instar larval gut labeled for the *amon* protein. The protein localizes to the midgut endocrine cells (inset) in the thicker regions of the midgut. Panel C (10×) shows the anterior portion of the midgut behind the proventriculus. Panel D (20×) shows a more posterior portion of the midgut located near the midgut–hindgut junction. Dashed box in C indicates area magnified in inset.

substrates of the *amon* protein. Using immunocytochemistry, we looked for co-localization of *amon* protein and candidate peptide hormone targets in the CNS.

The timing and behavioral events of larval molting are controlled by the peptide hormones eclosion hormone (EH), ecdysis triggering hormone (ETH), and crustacean cardioactive peptide (CCAP) (Mesce and Fahrbach, 2002). The localization of these three peptide hormones in *Drosophila* have been established (Horodyski et al., 1993; Park et al., 2002, 2003), and both ETH and CCAP contain potential dibasic processing sites (Park et al., 2002, 2003). In other insects, mature bioactive EH appears to be produced directly following cleavage of the signal sequence by signal peptidase (Horodyski et al., 1989; Wei et al., 2008; Zhang and Xu, 2006). The *Drosophila* EH pro-peptide includes a dibasic KR motif located 9 amino acids after the putative signal peptidase cleavage site (Horodyski et al., 1993). However, it has not been demonstrated that this potential processing site is used or is necessary for production of the mature bioactive *Drosophila* EH peptide. We used

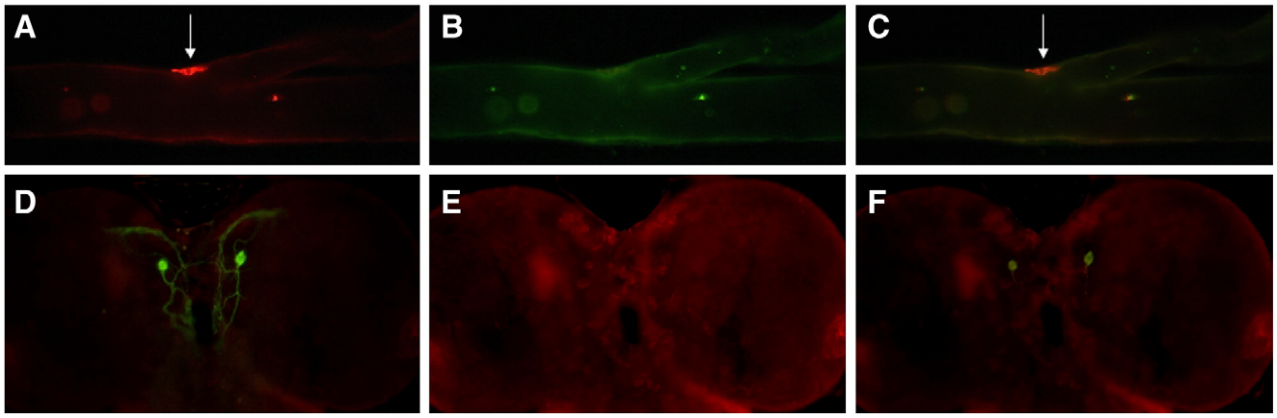


**Fig. 4.** The *amon* protein does not co-localize with ETH or EH. (A) Third instar larval trachea labeled using an antibody directed against ETH (Park et al., 2002). The arrow indicates the Inka cell. (B) The same trachea labeled using an antiserum directed against the *amon* protein. (C) Merged image (40×). (D) Third instar CNS of a larva carrying an EH-GAL4 driver (McNabb et al., 1997) and a UAS-mCD8-GFP reporter (Lee and Luo, 1999). (E) The same CNS as in (D) labeled for *amon* protein. (F) Merged image.

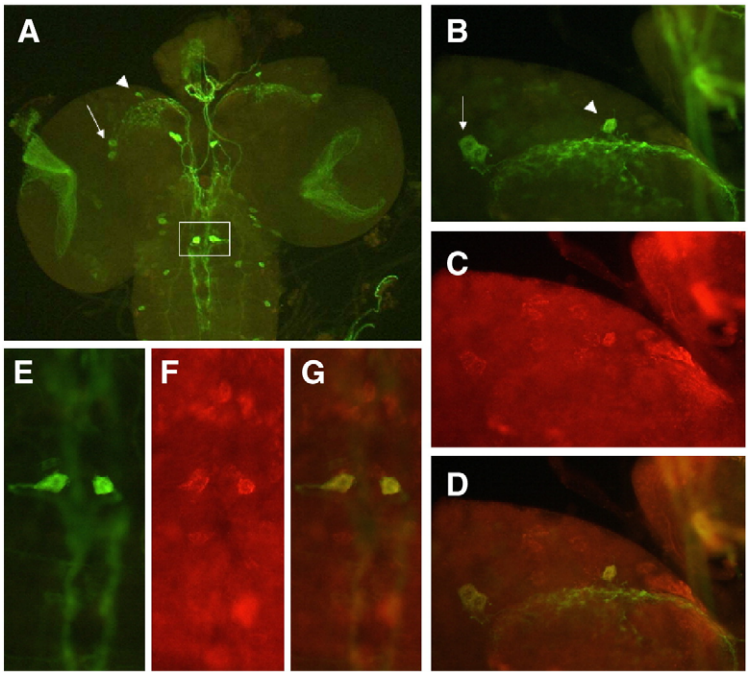


**Fig. 5.** The *amon* protein localizes to CA and CC-innervating neurons. (A) Third instar CNS of a larva carrying the Kurs-21 GAL4 driver (Siegmund and Korge, 2001) and the UAS-mCD8-GFP reporter (Lee and Luo, 1999). GFP signal is seen in the CA-LP1 (arrowhead), the CA-LP2 (arrow), and CC-MS2 (box) neurons. (B) A separate third instar CNS carrying the Kurs-21 GAL4 driver and the UAS-mCD8-GFP driver. (C) The same CNS as in (B) labeled with antiserum against the *amon* protein. (D) Merged image of (B) and (C). (E). Third instar CNS larva carrying the Kurs-21 GAL4 driver and the UAS-mCD8-GFP driver. GFP signal is seen in the CC-MS2 cells. (F). The same CNS as is (E) labeled for the *amon* protein. (G) Merged image of (E) and (F).

an antibody against ETH as well as EH and CCAP-cell GAL4 drivers and GFP reporters to identify ETH, EH, or CCAP-expressing cells in the brains of wild-type larvae. We then asked whether the *amon* protein was co-expressed in these cells (Fig. 4). As seen in Figs. 4A–C, the *amon* protein and ETH do not co-localize to the epitracheal Inka cell. Similarly, *amon* protein does not co-localize with EH in the two ventromedial EH neurons (Figs. 4D–F), nor does it localize to the CCAP-expressing cells (data not shown). Our results with ETH and CCAP confirm the previous study of Park et al. (2004) and together, these results suggest that ETH, EH, and CCAP are not direct substrates of the *amon* protein.

The lack of abdominal differentiation in both $amon^{C241Y}$ and $amon^{Q178st}$ mutant pupae (Figs. 1C,D, Table 2) resembles that seen in late third instar larvae and pre-pupae treated with excess amounts of the terpenoid juvenile hormone (JH) and its mimics (Ashburner, 1970; Madhavan, 1973; Zhou and Riddiford, 2002). Because the release of JH from the corpus allatum (CA) is inhibited by the peptide hormone allatostatin (Nijhout, 1994), animals treated with excess JH and animals lacking an active allatostatin peptide hormone could be predicted to display similar phenotypes. Recently Siegmund and Korge (2001) identified a GAL4 driver that strongly identifies three neurons innervating the CA, and is weakly expressed the VM and

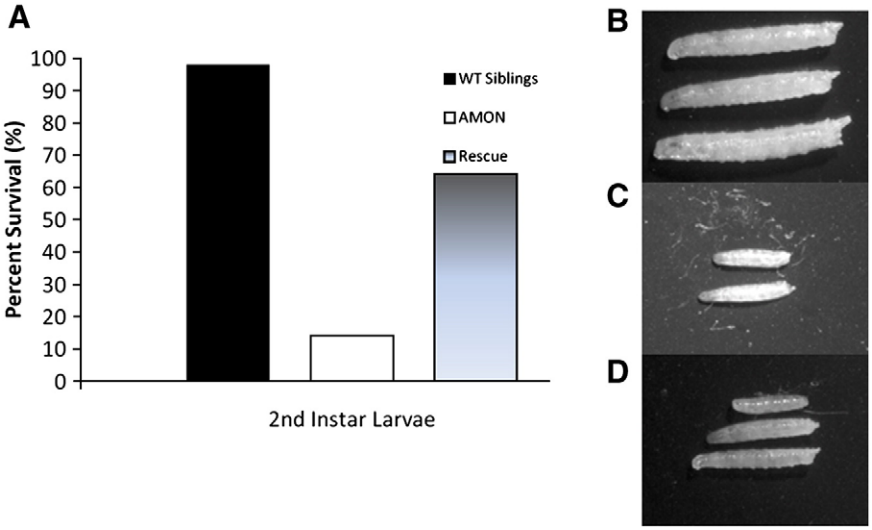


**Fig. 6.** *amon* expression in cells defined by the Kurs-21 GAL4 driver of an *amon* mutant is sufficient to partially rescue the molting and growth defect seen in *amon* mutants. (A) Percent survival to the second instar larval stage is shown for *amon* mutants (*yw; hs-amon/+; Df(3R)Tl-X e/kurs-gal4, amon*$^{C241Y}$, white bar), their control siblings (*yw; hs-amon; Df(3R)Tl-X e* or *kurs 21-gal4, amon*$^{C241Y}$*/TM3 Sb Ser y+ e*, black bar), and rescued *amon* mutants (*yw; hs-amon/uas-amon; Df(3R)Tl-X e/kurs 21-gal4, amon*$^{C241Y}$, hatched bar). $N = 100$ animals per genotype. In addition to increased survival, rescued *amon* mutants show an intermediate size (D) between control siblings (B) and *amon* mutants (C). Representative larvae are shown.

CCMS 1. Because these three neurons account for all observable synapses on the CA using synapse-specific antibodies, Siegmund and Korge argue that these are the only neurons in *Drosophila* that form synapses on the CA (Siegmund and Korge, 2001) making them candidates for allatostatin expression. We used the Kurs-21 driver (Fig. 5A) and a GFP reporter to identify these neurons and then examined them for *amon* protein localization.

Figs. 5B–D show that the *amon* protein localizes to the three CA-innervating cells, CA-LP1 and CA-LP2. In addition, the *amon* protein localizes to two cells also identified by the Kurs-21 GAL4 driver known as CC-MS2 (Figs. 5E–G) which innervate the corpora cardiaca (CC) portion of the ring gland. It is therefore tempting to speculate that *amon* activity in cell types that innervate the CA and CC may influence the regulatory activities of these two important endocrine signaling centers.

To examine the functional consequences of restoring *amon* expression in the CA-LP1 and CA-LP2 cells in an *amon* mutant background, we expressed a *uas-amon* construct in these cells using the Kurs-21 GAL4 driver. Fig. 6A shows that *amon* function in these cells is sufficient to partially rescue developmental progression. Only 14% of first instar *amon* mutant larvae successfully complete the first to second instar larval molt and emerge as second instar larvae. In contrast, when *amon* function is restored in the CA- and CC-innervating neurons in an *amon* mutant background, 64% of *amon* mutant larvae complete this molt and emerge as second instar larvae (Fig. 6A). Restoring *amon* expression to the CA- and CC-innervating cells is also sufficient to partially rescue growth in *amon* mutants (Figs. 6B–D). Rescued *amon* mutant larvae (Fig. 6D) are intermediate in size compared to *amon* mutant (Fig. 6C) and control sibling larvae (Fig. 6B). Thus, restoring *amon* function in the cells defined by the Kurs-21 GAL4 enhancer trap line is sufficient to partially rescue the molting and growth defects exhibited by *amon* mutants.

## Discussion

### *amon is required for pupal development*

Analysis of loss of function *amon* mutations has shown that *amon* is required during early larval development and for the completion of the first to second instar larval molt (Rayburn et al., 2003). Here we have used a conditional rescue system to show that *amon* is also required for successful completion of pupal development and eclosion of the adult fly (Table 1). The predominant phenotypes of two *amon* mutant alleles in which *amon* expression was removed at the late third instar larval stage indicate a requirement for *amon* for behavioral events characterizing early stages of pupal development (Fig. 1, Table 2) and suggest that the developmental events of metamorphosis can be uncoupled.

The arrested *amon* mutant pupae fail to undergo head eversion (Fig. 1), an event that occurs in wild-type animals at 12–14 h after pupariation (Bainbridge and Bownes, 1981), yet they complete processes such as eye pigmentation and head bristle formation (data not shown) that typically take place only after successful head eversion. Interestingly, despite the failure to complete head eversion and leg and wing extension, two of the earliest events of pupal development, the thoracic segment of these animals appears to develop normally as evidenced by its pigmentation and bristle formation (Fig. 1). Similarly, in a majority of the $amon^{C241Y}$ mutant pupae (58%) and a significant percentage of the $amon^{Q178st}$ mutant pupae (27%), the abdominal segment fails to differentiate despite the developmental progression of the anterior segments (Table 2, Fig. 1). In addition, a small subset of arrested $amon^{C241Y}$ pupae (15%) fail to anteriorly displace the mid-body gas bubble, an event that typically happens prior to head eversion, yet they display abdominal differentiation. These observations suggest that the head, thoracic, and abdominal segments of the fly respond to endocrine cues independently of one another and that interruption of these signals can cause an uncoordinated pattern of metamorphosis.

### *The amon protein localizes to the nervous system but does not co-localize with known neuropeptides governing molting and metamorphosis*

The *amon* protein localizes to cells in the central nervous system and gut in third instar animals (Fig. 3). To identify potential *amon* protein substrates, we compared the localization of three neuropeptides involved in molting and metamorphosis, CCAP, ETH, and EH (Park et al., 2003; Ewer et al., 1997; McNabb et al., 1997; Park et al., 2002) to that of the *amon* protein. Phenotypic similarities between *amon* and ETH mutants (Park et al., 2002) and *amon* mutant pupae and pupae lacking the CCAP neurons (Park et al., 2003) suggested a relationship between *amon* and these peptide hormones. However, the *amon* protein does not co-localize with CCAP (data not shown), ETH, or EH (Fig. 4) arguing against a direct enzyme–substrate relationship between the *amon* protein and these neuropeptides. It is interesting to note, however, that *amon* transcripts were detected in the Inka cells by Siekhaus and Fuller (1999), but that another group (Park et al., 2004) using a different antiserum against the *amon* protein (Hwang et al., 2000) have also determined that the *amon* protein does not localize to the Inka cells, suggesting possible post-transcriptional regulation of the *amon* mRNA. This same group also examined *amon* protein's localization in relation to the CCAP-expressing cells and observed the same lack of co-localization we report here (Park et al., 2004). Together, these results suggest that additional, as yet unidentified peptide hormones are involved in the regulation of larval molting and metamorphosis. Such a conclusion is supported by a study of the relationship among EH, ETH, and CCAP which suggests that exogenous ETH's ability to elicit pre-ecdysis requires the EH cells and that EH may act independently of CCAP to elicit ecdysis (Clark et al., 2004).

Western analysis shows that the *amon* protein is present in all stages of development (Fig. 2). We note that the band recognized by our antiserum on Western blots does not migrate with a size similar to that reported for the *amon* protein in a previous study in which two bands migrating at 80 and 75 kDa were detected from extracts of third instar larvae and the medium of S2 cells expressing *amon* and *d7B2* (Hwang et al., 2000). We cannot currently explain the apparent size differences between these studies but note that the 97 kDa band detected here is competed by addition of *amon* peptides and not by a non-specific peptide competitor and that heat-shock induced expression of an *amon* cDNA leads to leads to increases in abundance of the 97 kDa band (data not shown). It is possible that differences in the apparent mobility of the *amon* protein result from differing polyacrylamide gel electrophoresis conditions in the two studies.

### *Identification of cell-type specific requirements for amon*

Although *in vivo* mutational studies are critical to understanding the physiological roles played by SPCs (Furuta et al., 1997; Kass et al., 2001; Rayburn et al., 2003), the fact that these proteases have multiple processing targets creates difficulties in interpretation of phenotypes seen in mutants completely lacking function for a given SPC. Here we have begun to dissect the signaling contribution of subsets of neuronal cells to normal *Drosophila* development by restoring *amon* expression to these cells in an *amon* mutant background. We find that expression of *amon* using the Kurs-21 GAL4 driver partially rescues larval growth and molting defects exhibited by *amon* mutants (Fig. 6). This result ties *amon* function in cells defined by the Kurs-21 GAL4 driver to the regulation of larval molting and growth and suggests that the Kurs-21 cells may produce a processed peptide hormone signal that functions in these pathways. Because subsets of Kurs-21 cells make synaptic connections to either the CA or the CC, in the future it will be

interesting to explore whether the rescuing activity demonstrated here acts *via* the CA or CC, or both, and whether this effect intersects the known signaling pathways controlling ecdysis and growth.

Previous genetic analysis of the peptide hormone processing enzyme PHM, which acts to α-amidate C-terminal residues of secretory peptides, has shown the importance of secreted peptide hormones in the modulation of developmental transitions and highlighted the potential of genetic methods in dissecting the contribution of secreted peptides to normal developmental progression (Jiang et al., 2000). The rescue approach that we describe here is a flexible genetic tool that can be used to correlate *amon* function in specific neuronal cells with the control of normal aspects of development and physiology as initially inferred from *amon* loss of function genetic studies and to ask whether and how *amon* contributes to the regulation of peptide hormone signaling. A powerful complementary approach to determine cell types in which *amon* function is required for normal growth and development will be the reduction of *amon* function in specific neuronal cell types using RNA inactivation. Numerous peptide hormones possessing potential dibasic cleavage sites have been identified in *Drosophila* (Hewes and Taghert, 2001). Ultimately, the genetic techniques described above may identify cells in which these known peptide signals are used in new ways and have the potential to lead to the identification of novel peptide signals.

The phenotypic and localization data presented here suggest that the *amon* protein functions in a wide variety of neuroendocrine cell types to process and activate a diverse suite of peptide hormones that coordinate larval and pupal development. In the future, we hope to identify cell types in which *amon* function is required for control of specific physiological, behavioral, or developmental events through cell-type specific rescue and inactivation experiments. A second important challenge for the future is biochemical identification of direct proteolytic substrates of the *amon* protein.

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